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Extranodal NK/T-cell lymphoma, nasal type without evidence of EBV infection

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Abstract. Extranodal natural killer/T cell lymphoma-nasal type (EN-NK/T-NT) is extremely rare in Western countries; however, it is the most common subtype of peripheral T cell lymphoma in China. Despite this, there are a limited number of clinicopathological research studies on Epstein-Barr virus (EBV)-negative EN-NK/T-NTs. EBV-negative EN-NK/T-NT is a rare disease type, which has not been fully investigated. If other diagnostic criteria are met, such as the lesions being located predominantly in the upper aerodigestive tract, the presence of angiocentricity or angioinvasion, necrosis and expression of NK/T-cell phenotype, EN-NK/T-NT may be diagnosed, even if EBV is negative. In the present study, 99 cases of EN-NK/T-NTs were analyzed retrospectively, among which seven cases were EBV-negative EN-NK/T-NTs and selected for further investigation. In addition, the present study reviewed previously published research into EN-NK/T-NT, highlighting that EBV-negative EN-NK/T-NT is rare and that its geographical distribution is mainly in countries in Asia, Central America and South America. Patients with EBV-negative EN-NK/T-NT were all of Chinese ethnicity, with a median age of 32 years and primarily female. Furthermore, these patients shared similar clinicopathological characteristics (such as the tumor occurring mainly in the upper aerodigestive tract, the presence of vascular destruction, necrosis and cytotoxic phenotypes) to patients with EBV-positive EN-NK/T-NT. Immunohistochemistry and molecular analysis results indicated that tumor cells were primarily of NK or cytotoxic T origin; however, EBV-encoded small RNAs were not detected in any of these cases. Among the immunochemistry markers, T-bet was statistical significantly different between EBV-positive and -negative cases. Fluorescence *in situ* hybridization was also performed in two EBV-negative cases, including one case with a co-deletion of 6q21 and PR/SET domain 1 genes. There was only available follow-up data in 3/5 patients who survived for 37-113 months (median, 40 months). As EN-NK/T-NT can be diagnosed, even when EBV is negative, awareness of this subtype may prevent misdiagnosis or delayed diagnosis.

Introduction

Extranodal natural killer/T cell lymphoma-nasal type (EN-NK/T-NT) is a type of lymphoma which primarily occurs in the nasal cavity and nasal pharynx (1-5). Furthermore, the incidence rate is higher in Asian countries compared with that in Western countries (1-4). In a study published in 2017, the incidence rate in Asia and Latin America (10% of all non-Hodgkin's lymphoma) was reported to be higher than that in Europe and North America (<1%) (5). As shown in our previous study, EN-NK/T-NT is the most common subtype of peripheral T cell lymphoma in China (1). EN-NK/T-NT occurs primarily in adult males, and often presents as a localized disease involving the nasal cavity or its surrounding structures; furthermore, it is characterized by vascular destruction, necrosis and a cytotoxic immunophenotype (1-4). EN-NK/T-NT is named 'NK/T' as opposed to 'NK' primarily as the majority cases appear to be true NK-cell tumors, a few cases can manifest as cytotoxic T-cell immunophenotype (2,3). Epstein-Barr virus (EBV) infection can be detected in the tumor cells in the form of clonal epistasis, which suggests that this virus might have an important role in the pathogenesis of lymphoma, independent of race and geographical distribution (2,3).

The association between EN-NK/T-NT and EBV infection was first described in 1988 and assists in the diagnostic and pathological understanding of the disease (6). Harabuchi *et al* (7) and Ho *et al* (8) identified an association between EN-NK/T-NT and EBV using Southern blot hybridization for EBV DNA. Moreover, a seminar co-sponsored by the University of Hong Kong and the Society for Hematopathology held on October 9, 1994, discussed the definition, diagnosis, differential diagnosis and epidemiology of angiocentric lymphomas occurring in the nose and other

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Abbreviations: EN-NK/T-NT, extranodal natural killer/T-cell lymphoma-nasal type; EBV, Epstein-Barr virus; EBER, EBV-encoded RNA; ISH, *In situ* hybridization; FISH, fluorescence *in situ* hybridization; TCR, T cell receptor; OS, overall survival

Key words: EBV, EBER, EN-NK/T-NT, ISH, T-bet, PR/SET domain 1

extra-nodal sites, including the skin, subcutis and gastrointestinal tract (9). The term 'nasal T/natural killer (NK) cell lymphoma' identifies its association with EBV, which assists in the clinical diagnosis of the disease; however, the term lacks a definition of lineage. Thus, the term EN-NK/T-NT has been classified as a clinicopathological disease, which is associated with EBV by the World Health Organization (WHO) since 2001 (2,3,10,11). In the WHO 2008 classification, the presence of EBV was included in the definition of the disease by evaluating the EBV-encoded small RNA (EBER), and EBV may be associated with the pathogenesis of the disease (2). Positive identification of EBER is considered to be a requisite for the diagnosis of this disease, and the detection of EBER using *in situ* hybridization (ISH) in paraffin-embedded samples remains the gold standard for EBV detection, as expression of latent membraneprotein1 (LMP1) is inconsistently detected in EBV-positive tumors (2,3,11). Furthermore, the combination of immunostaining of CD3, CD20, CD56, TIA1 and granzyme B, and T-cell receptor (TCR) gene rearrangement analysis is required for the accurate diagnosis of T cell and NK cell lymphomas (1-3,10).

EN-NK/T-NT is associated with EBV infection, which is different from other types of mature T/NK cell lymphoma, including peripheral T-cell lymphoma, not otherwise specified, anaplastic large cell lymphoma, adult T-cell leukemia/lymphoma and hepatosplenic T-cell lymphoma (1-3,10). Previous studies have revealed that EBER can be detected in nearly all EN-NK/T-NT cases (1-3,10). Moreover, there is a higher incidence rate of EBV infection in EN-NK/T-NT in Asian compared with Western countries; however, cases without detectable EBV may still be suspected of diagnosis (2,3,12). Classic features of EN-NK/T-NT have been widely accepted and include patients being from Asian and Central and South American countries, the tumor being located in the upper aerodigestive tract, being morphologically characterized by vascular destruction and necrosis, expressing NK or T cell markers, and ≥1 cytotoxic molecules, consistent association with EBV and germline TCR gene; however, controversy remains regarding atypical or discordant cases, such as the tumor occurring in other extranodal sites (including the skin, subcutis, testes and gastrointestinal tract), the tumor cells being small and mixed with inflammatory infiltratory cells without angiodestruction characteristic, and demonstrating an atypical phenotype (such as being CD3-, CD56- or TIA-1-negative and CD30-positive, and having an aberrant CD20 expression), and particularly the absence of EBV (9,13).

A previous seminar by the Society for Hematopathology/European Association for Hematopathology on the NK/T cell malignant tumors (13) discussed three cases with typical location or immunotype of EN-NK/T-NT, but with EBV-negative expression. Moreover, the seminar assessed challenges faced with the classical definition of the disease; however, the lymphomas classification involving NK features remains controversial. Data on patients with EBV-negative EN-NK/T-NT are limited. The present study identified seven EBV-negative cases from a total of 99 EN-NK/T-NT, and retrospectively analyzed the clinicopathological and molecular characteristics of the lymphoma in China. Furthermore, the results were also compared with that in EBV-positive cases.

Materials and methods

Patients and tissue samples. The pathology archives at the Department of Pathology, First Hospital of Peking University, as well as the data files, were searched between January 2001 and December 2016, and 99 EN-NK/T-NT cases were identified. In addition, seven patients with EBV-negative EN-NK/T-NT were further analyzed, retrospectively, for clinical information, including age, sex, location (including the upper aerodigestive tract and others), Ann Arbor stage (14), treatment (chemotherapy and/or radiotherapy) and survival status. The follow-up data were available for 62 patients, including 5 EBV-negative EN-NK/T-NT patients. Histologic sections were stained with hematoxylin and eosin, and all the sections in the database were reviewed by three pathologists blinded to the study. The pathologic diagnosis criteria were based on the 2001, 2008 and 2016 WHO classification: i) Patients presenting with extranodal/upper aerodigestive tract lesion; ii) tumor cells were evaluated for the presence of cytological features, angiocentricity or angioinvasion, necrosis and inflammatory cells; iii) immunophenotyping, including the expression of CD3, CD56 and cytotoxic molecules (TIA1 and granzyme B) in the absence of B-cell markers (CD20); and iv) EBER-positive expression using ISH (2,3,10). An angiocentric pattern could only be assessed in 84 patients and necrosis in 91 patients due to sampling, as many nasal biopsies were small, and the tumor cells may have been deformed or degenerated.

Immunophenotypical analysis. Immunohistochemical staining was observed under a light microscope with x400 magnification. Due to the extremely limited samples, it was not possible to analyze all the markers on the same sample simultaneously. Immunohistochemistry (IHC) was performed on a total of 99 samples using 4-μm thick sections from representative formalin-fixed (using 10% formalin at room temperature for 24 h) and paraffin-embedded tissue blocks, using the Dako EnVision detection kit (Dako; Agilent Technologies, Inc.). Briefly, before dewaxing, the tissue section was heated to 65˚C for ten min to remove the wax. The slides were subsequently washed twice with xylene for dewaxing for 10 min, then dehydrated in an ethanol descending gradient series (100, 100, 95,80 and 70%, for 2 min each time), and washed with distilled deionized water. Next, the slides were washed with PBS (5 times, 10 min each time), and the tissue sections underwent heat-induced antigen retrieval in EDTA-Tris (pH 9.0) at 97˚C for 20 min (PT Link; Dako; Agilent Technologies, Inc.). After the samples were washed with PBS for an additional 10min, 3% hydrogen peroxide was used to treat the samples for 10 min, followed by an additional wash with PBS for 5 min. Tissues were subsequently probed with primary antibodies for 1 h at room temperature. The following primary antibodies were used: Anti-CD3 (cat. no. LN10; 1: 50-100 depending on the tissue samples; OriGene Technologies, Inc.), anti-CD56 (cat. no. UMAB83; 1:100; OriGene Technologies, Inc.), anti-CD20 (cat. no. L26; 1:100; Dako; Agilent Technologies, Inc.), anti-TIA-1 (cat. no. 2G9A10F5; 1:100; OriGene Technologies, Inc.), anti-granzyme B (cat. no. EP230; 1: 50; OriGene Technologies, Inc.), anti-T-bet (cat. no. H-210; 1:100; Santa Cruz Biotechnology, Inc.) and ETS1 (cat. no. C-4; 1:100; Santa

Cruz Biotechnology, Inc.). The tissue sections were additionally washed with PBS (3 times, 5 min each time) and then probed for 20 min with a secondary antibody conjugated to horseradish peroxidase (cat. no. PV-6000-D; 1: 500; OriGene Technologies, Inc.) at 37˚C for 30 min. An additional PBS wash (3 times, 5 min each time) was subsequently performed, and the chromogenic 3,3'-diaminobenzidine mixture (OriGene Technologies, Inc.) was used to stain the samples at room temperature for 5 min. Then, the samples were dehydrated with an ascending ethanol series (75, 95, 100 and 100%) and washed with xylene, and natural gum was used to seal the samples.

The staining results were semi-quantitatively assessed using the following criteria: i) -, Positive cell ≤5%; ii) 1 +, positive cell 6-20%; iii) 2 +, positive cell 21-50%; and iv) 3 +, positive cell >50% (15). Positive and negative controls were used. From the aforementioned pathology archives, normal lymph nodes were used as the positive control for CD3 and CD20 expression, and EN-NK/T-NT samples with definite diagnosis were used as the positive control for CD56, TIA1, granzyme B, T-bet and ETS1 expression. PBS was used as the negative control.

In addition, immunohistochemical analysis was performed for anti-PR/SET domain 1 (PRDM1) (cat. no. C14A4; 1:100; Cell Signaling Technology, Inc.). Nuclear staining of PRDM1 in >10% of tumor cells was interpreted as positive. PRDM1 staining was semi-quantitatively assessed according to the follow criteria: i) -, No positive cell or positive cell <10%; ii) 1+, positive cell 10-≤50%; iii) 2+, positive cell >50-100%). For the negative control reactions, PBS was used (16). Analysis of the IHC results from the database was performed by three pathologists blinded to the study. Due to some samples being of poor quality, the results of some markers were lost.

ISH analysis. All cases were tested for EBER using ISH according to manufacturer's instructions. The probe for EBER-1 was supplied by OriGene Technologies, Inc. To assess staining, an EBV-positive nasopharyngeal carcinoma sample from our tissue bank was used as a positive control. Tumor nuclei stained with brown granules were interpreted as positive. The percentage of positive tumor cells was semi-quantitatively estimated as the standard in IHC. The seven EBV-negative cases were assessed two times for the 2 consulted cases and three times for the other 5 cases by repeat assays.

Molecular analysis for TCR gene rearrangement. TCR gene rearrangement was investigated in 7/99 cases, including 2 EBV-positive cases and 5 EBV-negative cases. TCR gene rearrangement was not performed for the remaining two EBV-negative consulted cases due to limited paraffin blocks. DNA was extracted from paraffin-embedded tissue samples using a Qiagen DNeasy blood and tissue kit (Qiagen GmbH), according to the manufacturer's instructions. TCR-γ (TCRG) and TCR-β (TCRB) chain clonality analysis was performed using PCR with the Identi Clone T-cell clonality assays (Invivoscribe, Inc.). The TCRG/B gene primers and the PCR protocols were designed according to a previous study (17). The PCR products were analyzed via 10% polyacrylamide gel electrophoresis, stained with 1 μg/ml ethidium bromide and observed under an ultraviolet illuminator. The results were interpreted following the manufacturer's instructions.

Fluorescence in situ hybridization (FISH) analysis. A total of 2 EBV-negative EN-NK/T-NT cases, from limited specimens of paraffin-embedded samples, were analyzed using FISH to detect gene aberration following the manufacturer's instructions. The DNA probes 6q21 (length, 409 kb) and PRDM1 (length, not available) FISH were used to detect the deletion of these two genes (Empire Genomics LLC). Slides were prepared and the results were analyzed as previously described (16).

Statistical analysis. The association between EBV expression and the clinicopathological characteristics of the patients, including age, sex, primary sites, Ann Arbor stage, angiocentricity and/or angioinvasion, necrosis and the expression levels of IHC markers (CD56, TIA1, granzyme B, T-bet and ETS1) were analyzed using either Fisher's exact or χ^2 tests. The clinicopathological features of EBV-positive and -negative cases, which occurred in the upper aerodigestive tract were also compared using either Fisher's exact or χ^2 tests. Overall survival (OS), which was defined as the day of initial diagnosis to the day of mortality due to any cause or the last follow-up, was determined using the Kaplan-Meier method and the comparison of differences between the OS of the EBV-positive and -negative groups was evaluated using the log-rank test. All the data are presented as number of cases, and all statistical analyses were performed three times using SPSS software (v23.0; IBM Corp.). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Clinical data. Of the 99 patients with EN-NK/T-NT, there were 57 men and 42 women (male:female ratio, 1.6:1). The median age was 43 years (range, 8-88 years). For one patient, the biopsy location and follow-up information were not available, leaving a total of 98 biopsies. The most common biopsy sites for initial diagnosis included the upper aerodigestive tract, which accounted for 75.5% (74/98) of cases, followed by 24.5% (24/98) of non-upper aerodigestive tract involvement, including the skin, lymph node, gastrointestinal tract and uterine cervix. There were clinical stage data for 64 patients, in which 23 patients (35.9%) presented at stage I-II, whilst 41 patients (64.1%) were stage III-IV, due to bone marrow and/or liver involvement. Furthermore, 30/58 patients (51.7%) showed B symptoms (fever, malaise and weight loss) on disease presentation.

The clinical characteristics of the seven patients with EBV-negative EN-NK/T-NT (median age, 32 years; age range, 8-59 years) are summarized in Table I. The results indicated a high female predominance (male:female ratio, 1:6). In addition, there was a significant difference in the sex of the patients between EBV-positive and -negative expression ($P=0.045$; Table II). The initial involvement sites of the seven patients were all in the upper aerodigestive tract, and 5/7 patients were diagnosed at Ann Arbor stage I while three patients had accompanying B symptom (Table I). There were no significant differences in age, involvement sites and stage between patients with EBV-positive and -negative expression ($P>0.05$; Table II).

Histology results. The histological examination of all the 99 extranodal/non-nasal lesion biopsy specimens revealed

Table I. Summary of Clinical findings of EBV negative EN-NK/T-NT.

Case no.	Age, years	Sex	Primary site	Ann Arbor stage	Therapy	Time, months	Survival
1	36	Female	Nasopharynx	IA	NA	NA	NA
2	31	Male	Nasal septum posterior extremity	IVB	ND	1	Died of disease
3	24	Female	Right tonsil	IA	R	113	CR
4	59	Female	Hard Palate	IA	NA	NA	NA
5	8	Female	back of the tongue	IVB	C	9	Died of MOF
6	32	Female	Skin of the left ala nasi	IB	C+R	40	CR
7	54	Female	Nasal cavity	IA	C+R	37	CR

C, chemotherapy; R, radiotherapy; CR, complete remission; MOF, multiple organ failure; NA, not available; ND, not done.

similar histological characteristics (data not shown). For example, the morphological lineage of tumor cells was extensive, characterized by mixed cell types of small, medium to large size. Furthermore, there was irregular nuclear morphology, chromatin granules, inconspicuous or small nucleoli and minimal-to-medium cytoplasm. In addition, there was a variable magnitude of inflammatory cells, including small lymphocytes, histiocytes, plasma cells, eosinophils and neutrophils. An angiocentric and/or angioinvasion pattern was also observed in 43/84 patients (51.2%) and necrosis of tumor tissue in 77/91 (84.6%) cases (data not shown).

Among the seven EBV-negative EN-NK/T-NT cases, epidermotropism, which was characterized by the invasion of tumor cells into the glandular epithelium or the surface mucosa, was observed in 5/7 cases (Fig. 1A). In addition, an angiocentric and/or angiodestructive infiltration was found in 1/7 (14.3%) cases, and necrosis was observed in 4/7 (57.1%) cases (Fig. 1A; Table III). However, no significant differences were demonstrated in the morphological features between EBV-positive and -negative cases (Table II).

Immunophenotypical results. All 99 cases of EN-NK/T-NTs had negative and positive staining for CD20 and CD3 expression, respectively. There was also positive expression for CD56 in 81/92 cases (88%), TIA-1 in 94/97 cases (96.9%) and granzyme B in 89/95 cases (93.7%). Furthermore, there were more patients who were positive for T-bet than for ETS-1, 93/97 (95.9%) and 70/92 (76.1%), respectively. In addition, PRDM1 expression was observed in 6/23 cases (26.1%).

All the seven cases of EBV-negative EN-NK/T-NTs were CD3-positive (7/7; 100%; Fig. 1B) and CD20-negative (7/7; 100%). In addition, more than half the patients had positive expression for CD56 (7/7; 100%; Fig. 1C), TIA1 (7/7; 100%; Fig. 1D), granzyme B (6/7; 85.7%; Fig. 1E), T-bet (5/7; 71.4%; data not shown) and ETS (16/7; 85.7%; data not shown). The levels of expression for the afore mentioned markers are shown in Table III. There was weak positive expression for PRDM1in only two cases (2/6; 33.3%). Furthermore, all the immunostaining results were similar with those found in the EBV-positive cases (Table III). Among the aforementioned markers, a significant difference in the expression of T-bet between EBV positive and negative expression was found (P=0.015; Table III).

EBV in situ hybridization. The results indicated that the tumor cells in 92/99 EN-NK/T-NT cases (92.9%) were positive for EBER mRNA using ISH, and the seven cases were EBER-negative (Table II; Fig. 1F). Additionally, the present study reviewed the literature and identified that there were indeed some EBV-negative EN-NK/T-NT cases, most of which were published in the form of case reports or small series (Table IV).

Genotype results. TCR gene rearrangement was detected in only 7/99 EN-NK/T-NT cases at diagnosis, of which 6/99 (6.1%) had germline TCR gene rearrangements. However, 5/7 (71.4%) EBV-negative cases all had germline TCR gene rearrangement, thus suggesting an origin in the NK-lineage (Table II). FISH detection of 6q21 and PRDM1 was performed in only two EBV-negative EN-NK/T-NTs specimens. In one case, both 6q21 and PRDM1 genes were deleted, while in the other case neither gene was abnormal (Table II).

Therapy, outcome and statistical analysis. In total, 62/99 patients had available follow-up data and the median OS was 22 months (range, 1-147 months). Overall, 43.5% (27/62) of patients were alive with or without lymphoma, while mortality occurred in 56.5% (35/62) of patients at the end of the follow-up period, due to tumor progression or related complications, as a result of drug toxicity, infection, systemic failure or other unknown reasons.

There was follow-up data for 5/7 patients with EBV-negative EN-NK/T-NT. The median OS was 37 months (range, 1-133 months) and the median follow-up time was 40 months (range, 37-113 months). Furthermore, 3/5(60%) patients were in remission following local radiotherapy or combined radiotherapy and chemotherapy. In total, mortality occurred in 2/5 patients (40%) due to rapid disease progression, both of whom died at stage IVB. In particular, 1 patient stopped treatment due to economic reasons and mortality occurred rapidly within 1 month following diagnosis. The remaining four patients were treated, including one patient receiving only chemotherapy, one receiving radiotherapy alone and two patients receiving both radiotherapy and chemotherapy. Progressive dissemination and chemo-resistance developed in 1 patient, and mortality occurred due to multi-organ failure within 9 months. Treatment and follow-up data are shown in

Table II. Differences in the clinical and pathological features between EBV-positive (n=92) and -negative cases (n=7).

Characteristic	EBV-positive, n	EBV-negative, n	P-value
Age, years			0.345
>60	21	0	
≤60	71	7	
Sex			0.045[a]
Male	56	1	
Female	36	6	
Primary sites			0.845
Nasal	69	6	
Extranasal	23	1	
Ann Arbor stage			0.495
I/II	20	5	
III/IV	39	2	
NA	33	0	
B symptom			0.999
Yes	28	3	
No	27	4	
NA	37	0	
Angioinvasive			0.183
Yes	42	1	
No	36	6	
NA	14	0	
Necrosis			0.121
Yes	73	4	
No	11	3	
NA	8	0	
CD56			0.581
0	11	0	
1+	12	2	
2+	23	1	
3+	39	4	
NA	7	0	
TIA1			0.508
0	3	0	
1+	11	2	
2+	23	1	
3+	53	4	
NA	2	0	
Granzyme B			0.315
0	5	1	
1+	18	3	
2+	25	1	
3+	40	2	
NA	4	0	
T-bet			0.015[a]
0	2	2	
1+	13	2	
2+	35	1	
3+	40	2	
NA	2	0	
ETS-1			0.097
0	21	1	
1+	28	3	
2+	24	0	
3+	12	3	
NA	7	0	
Survival			0.762
Alive	24	3	
Dead	33	2	
NA	35	2	

[a]P<0.05. NA, not available.

Table I. There was no significant difference in OS between the EBV-positive and EBV-negative cases (P=0.762; Fig. 2; Table III). Furthermore, EN-NK/T-NTs occurred in the upper aerodigestive tract, irrespective of EBV-positive or EBV-negative status, and were hypothesized to have similar clinicopathological features, except for gender (P=0.037; Table SI) and T-bet expression (P<0.001; Table SI).

Discussion

EN-NK/T-NT is an extranodal aggressive mature T or NK-cell lymphoma, which has been associated with EBV infection and cytotoxic tissue-destructive, and its incidence rate is higher in Asian countries compared with that in Western countries (1-4,10,12). A study published in 2017 reported that the incidence rate in Asia and Latin America (10% of all non-Hodgkin's lymphoma) was higher than that in Europe and North America (<1%) (5). However, EBV-negative EN-NK/T-NT is rare, even in Asia, where there is a high infection rate of EBV (3,18). The findings of the present study are similar with those of EBV-negative EN-NK/T-NT cases obtained through a literature review (Table IV). EBV-negative EN-NK/T-NT is rarely seen, most of which are case report or a small series of studies and the geographical distribution is mainly in Asian countries and other countries such as Central America and South America (Table IV). Whether these atypical cases should be defined as EBV-negative EN-NK/T-NT of the same disease or as an independent EN-NK/T-NT remains controversial, and the detailed clinicopathological features are limited.

In the etiology study of T-cell and NK-cell tumors, malignant transformation caused by EBV infection is a well-known factor in tumorigenesis, although the exact carcinogenic function of EBV remains unknown (2,3,11). Immunosuppression is an important risk factor for individuals who are susceptible to EBV infection-mediated malignant transformation (19). However, EBV has been associated with NK/T cell lymphoma in individuals who are not immunocompromised, suggesting that EBV infection may be opportunistic (20). In addition, most patients with persistent EBV infection will never develop

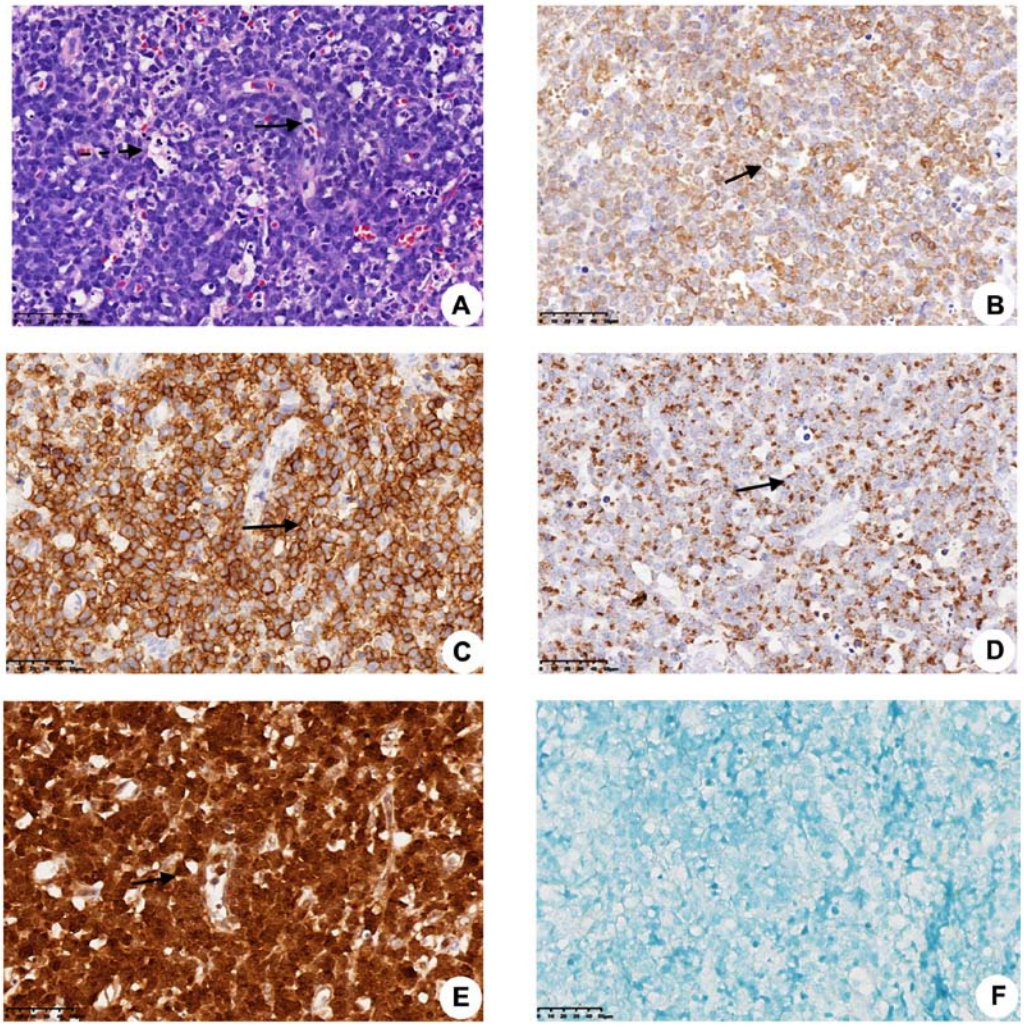

Figure 1. Staining images of EBV-negative extranodal NK/T cell lymphoma, nasal type. (A) High magnification showing the tumor cells are medium to large sized with irregular nuclei, granular chromatin and inconspicuous nucleoli, following hematoxylin and eosin staining. The black arrow indicates the blood vessels that are surrounded and infiltrated by tumor cells, and the dashed arrow indicates the nuclear debris. Immunohistochemistry identified strongly positive expression (3+, positive cell >50%) for (B) CD3, (C) CD56, (D) TIA-1 and (E) granzyme B. There was positive CD3 and CD56 expression at the cell membrane, while TIA-1 and granzyme B was positivity expressed in a granular pattern in the cytoplasm (arrows). (F) There was no positive expression of Epstein Barr virus-encoded small RNA in the nuclei of tumor cells using in situ hybridization. Magnification, x400.

EN-NK/T-NT, indicating that EBV does not function alone and that it is likely that host genetic and environmental cofactors or lifestyle differences are also implicated (11,21). Moreover, it is suspected that NK or T cells may be infected when the organism's immunity is reduced or immunosuppressed, and undergo malignant transformation, which may be caused by other pathogenic factors unrelated to EBV infection (2,11,19), such as the possibility of ethnic, geographic heterogeneity, genetic background and environmental cofactors, or lifestyle differences (11,21); however, this requires further investigation.

The present study described the clinicopathological features of seven patients with lymphoma of the upper aerodigestive tract, with the presence of neoplasms morphology and angioinvasion. Furthermore, these patients presented with necrosis and expressed the NK/T-cell phenotype (positive expression for CD3, CD56, TIA1 and granzyme B). TCR gene rearrangement analysis was not routinely performed due to the poor quality of specimens, including small sample size and/or wide necrosis areas. It was found that two cases had monoclonal TCR gene rearrangements and were therefore classified as T cell lineage, while the remaining cases were categorized into NK cell lineage. The diagnosis of EN-NK/T-NT was supported by the comprehensive analysis of clinicopathological features, immunophenotypic data and molecular results, but had a negative expression of EBV, which is inconsistent with the diagnostic criteria defined by the WHO (2,3,8). However, the results of the present study and the research shown in Table IV indicates that EBV-negative EN-NK/T-NT does exist, and EBV-negative results do not exclude the possibility of an EN-NK/T-NT diagnosis.

The existence of the EBV-negative cases requires further investigation to determine whether: i) EBV is required or solely acts as a 'passenger' in the oncogenic functions of EN-NK/T-NT (22); ii) there is another latent pattern of EBV expression that does not express EBER, or alternative oncogenic mechanisms other than infection of EBV, such as recurrent mutations in MLL2, BCOR, STAT3, JAK3, TP53 and KDM6A genes (23); iii) some patients with EBV-negative cytotoxic lymphoma with NK cell characteristics may lose EBV expression during clone amplification (24,25).

Table III. Summary of the pathology results in EBV-negative extranodal NK/T-cell lymphoma, nasal type cases.

Case no.	Angioinvasive	Necrosis	Protein expression							EBER	TCR	6q21 deletion	PRDM1 gene deletion
			CD3	CD56	TIA-1	GB	T-bet	ETS1	PRDM1				
1	No	No	1+	1+	1+	-	-	-	-	-	No	ND	ND
2	No	Yes	3+	1+	2+	2+	1+	1+	-	-	No	ND	ND
3	No	No	2+	3+	1+	1+	3+	3+	+	-	No	No	No
4	No	Yes	3+	3+	3+	1+	1+	1+	+	-	No	ND	ND
5	No	No	3+	3+	3+	1+	-	3+	-	-	No	Yes	Yes
6	No	Yes	3+	2+	3+	3+	3+	3+	ND	-	ND	ND	ND
7	Yes	Yes	3+	3+	3+	3+	2+	1+	-	-	ND	ND	ND

The immunostaining results of CD3, CD56, TIA1, GB, T-bet and ETS1 were semi-quantitatively evaluated as follows: - (<5% cells positive), 1+ (6-20% cells positive), 2+ (21-50% cells positive) or 3+ (>50% cells positive), while PRDM1 expression was semi-quantitatively evaluated as follows: - (no positive cell or positive cell <10%), 1+ (positive cell 10% to ≤50%), 2+ (positive cell >50% to 100%). EBV, Epstein-Barr virus; EBER, EBV-encoded RNA; TCR, T cell receptor; +, positive; -, negative; ND, not done; GB, granzyme B.

Table IV. Distribution of cases reported as EN-NK/T-NT without EBV infection in different studies or case reports.

Author, year	Country of residence	Number of cases (%)	(Refs.)
Harabuchi *et al*, 1996	Japan	2/18 (11)	(44)
Nakamura *et al*, 1997	Japan	5/32 (16)	(12)
Cuadra-Garcia *et al*, 1999	USA	1/14 (7)	(45)
Quintanilla-Martinez *et al*, 1999	Peru	1/28 (4)	(46)
Ko *et al*, 2000	Korea	6/46 (13)	(4)
Jung *et al*, 2001	Korea	4/13 (31)	(47)
Ohshima *et al*, 2002	Japan	3/9 (33)	(48)
Kim *et al*, 2003	Korea	10/35 (29)	(49)
Ko *et al*, 2004	Korea	11/49 (22)	(26)
Ng *et al*, 2004	Singapore	1/42 (2)	(50)
Miyazato *et al*, 2004	Japan	11/34 (32)	(51)
Tai *et al*, 2004	Malaysia	1/20 (5)	(52)
Cabrera *et al*, 2007	Chile	2/9 (22)	(53)
Matsuda *et al*, 2009	Japan	1/1 (100)	(54)
Teo *et al*, 2011	China	1/1 (100)	(22)
Bouchekioua *et al*, 2013	France	1/23 (4)	(24)
Kim *et al*, 2015	Korea	1/1 (100)	(55)
Tian *et al*, 2015	China	1/1 (100)	(56)
Nicolae *et al*, 2017	USA	7/7 (100)	(27)
Tsuyama *et al*, 2018	Japan	1/1 (100)	(23)
Zeng *et al*, 2017	China	22/56 (39)	(57)
Asif *et al*, 2019	USA	1/1 (100)	(43)

In the present study, all the patients with EBV negative EN-NK/T-NT were of Chinese ethnicity, which is consistent with previous studies in which EN-NK/T-NT occurs at a higher rate in Asian countries (1-4,10). The median age of the patients was 32 years, which is slightly younger compared with that in previous studies (2-4,10,12,23). Moreover, there was a higher number of females, which is in contrast with that in previous studies on EN-NK/T-NT, which found that males are more frequently affected (2-4,10,12). The present study compared the sex difference between EBV-positive and -negative cases,

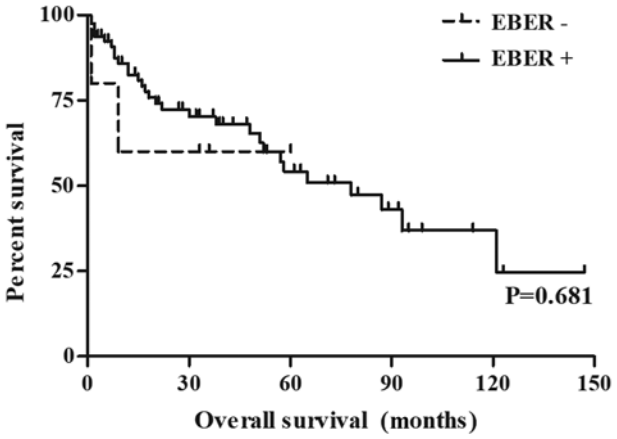

Figure 2. Overall survival rate of 99 patients with extranodal NK/T cell lymphoma, nasal type base and Epstein Barr virus expression. EBER, Epstein Barr virus-encoded small RNA; -, negative; +, positive.

and EBV infection was more likely in male patients compared with that in female patients, which was significant. However, the specific mechanism of this phenomenon has not been fully elucidated and requires further investigation, although it was hypothesized that the hormone levels of androgen and estrogen may have an effect on EBV infection. The results from the present study suggests that the upper aerodigestive tract was the most common site of involvement (75.5%), which is consistent with previous findings (1-4,10,12). Moreover, patients present with nasal obstruction, rhinorrhea and epistaxis due to mass or ulceration in the involvement sites (2,3,10,12).

A total of two patients presented with stage IV EN-NK/T-NT, and dissemination of the tumor cells to bilateral eyelids and to the bone marrow or liver. Furthermore, mortality occurred in one patient due to the disease at 1 month following diagnosis, while the other patient died due to multiple organ failure at 9 months following diagnosis; the mean OS of these two patients was 5 months. Moreover, patients with stage I EN-NK/T-NT had an improved survival rate. Previous studies have shown that the prognosis of patients in stage I was improved compared with patients at stage II and IV (2-4,12). However, the present study found no significant difference between clinical stage and survival rate. It has been reported that patients with EBV-negative EN-NK/T-NT have an improved response to chemotherapy and less aggressive phenotype compared with patients who are EBV-positive (12,26,27). Ko *et al* (28) reported that in EN-NK/T-NT cases, patients who were EBV-negative had a longer survival time compared with those who were EBV-positive. However, the follow-up data obtained in the present study could not be used to evaluate the prognostic influence of EBV infection, and the difference was not statistically significant, which may be due to the small sample size, consistent with the study by Nakamura *et al* (12). Thus, it was hypothesized that the prognosis of patients may be associated with other factors, such as advanced-stage disease (stage III or IV) and invasion of bone or skin (3,12), and not EBV infection alone.

The histological features found in the EBV-negative cases were similar with those in the EBV-positive cases. The cytological spectrum of EN-NK/T-NT is broad; neoplastic cells in the present study were predominantly medium size and inflammation was present. The histological characteristics are associated with angioinvasiveness and necrosis of tumor tissue (2,3,10). Furthermore, in the present study angioinvasion and necrosis in EBV-negative cases were 3.9 and 1.9 times lower compared with that in EBV positive cases, respectively. Kanno *et al* (29) found that EBV-infected lymphoid cells adhered to the vascular wall via cytokines, such as tumor necrosis factor (TNF)-α, interferon (IFN)-γ and interleukin (IL)-1β, and interacted with adhesion molecules, such as intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), on endothelial cells irrespective of neoplastic transformation, which subsequently initiated the destruction of vascular lesions in EBV-positive NK/T-cell lymphomas; the terms 'angiocentric' or 'angiodestructive pattern' are often used to describe these lesions (2,3,8). Moreover, CD56 can increase the ability of tumor cells to strongly adhere to and destroy blood vessel walls, which may also rely on cytokines (TNF-α and IFN-γ), resulting in angioinvasive and angiodestruction (29,30). Another previous study revealed that the upregulation of cytokines, including murine IFN-γ-inducible protein and monokine IFN-γ-inducible, was associated with the degree of necrosis (26). The cytotoxic granule proteins (TIA1 and granzyme B) may also affect angiodestruction and necrosis (29,30). Takeshita *et al* (30) found that EBV infection had a reduced effect on the histology of angiodestruction and necrosis. The present study identified no significant difference between angioinvasion or necrosis and EBV status, which is consistent with these previous studies, this may be related to the limited sample size, and more cases are required for further investigation.

It was demonstrated that all seven cases of EN-NK/T-NT had positive staining for CD3, CD56 and ≥1 of the cytotoxic molecules (7/7 for TIA-1 and 6/7 for granzyme B), and negative expression of CD20. Moreover, it was found that there was positive expression of CD3 in all of the seven cases; these neoplasms are hypothesized to arise from NK-like cytotoxic T-cells (2,8). T-bet and ETS-1 (5/7 for T-bet and 6/7 for ETS1) were also detected using immunohistochemistry in the tumor cells. Our previous studies have shown that T-bet and ETS-1, as transcription factors, serve an important biological role in lymphomagenesis (1,15). Furthermore, these transcription factors are upregulated in EN-NK/T-NT and are important markers in the diagnosis of EN-NK/T-NT (15). Lin *et al* (31) revealed that, in NK cells infected with EBV, microRNA-BART20-5p, which is encoded by EBV, inhibited the translation of T-bet, induced T-bet to upregulate p53 and inhibited p53 in invasive EN-NK/T-NT. Moreover, the results from the present study found a significant association between T-bet expression and EBV-positive and -negative cases, which indicates the interaction between T-bet and EBV to contribute to lymphomagenesis. As important synergistic factors, ETS-1 and T-bet regulate the terminal differentiation of NK and cytotoxic T cells, activate cytotoxic expression and stimulate the production of IFN-γ, which promotes lymphoma progression (15). The present study also found that these two transcription factors were highly expressed in seven EBV-negative EN-NK/T-NT cases (6/7, 85.7%) and both were markedly expressed in two cases. Thus, these transcription factors may serve a pathogenic role via non-EBV infection pathways (such as the JAK/STAT1 and JAK/STAT4 pathways)

and may be sensitive markers for EN-NK/T-NT (32). In addition, several cytokines and chemokines, including IFN-γ, IL-4, IL-5, IL-9, IL-10 and IL-13, produced by NK/T tumor cells can also form a network microenvironment, which promotes the expression of these two transcription factors (32,33). Moreover, these transcription factors can positively regulate the development of NK cells to serve a cytotoxic role, thus producing cytotoxic effectors that are associated with clinically aggressive features, including extensive destructive midfacial lesions, dissemination to various sites and potential complication by hemophagocytic syndrome (3,15). However, the exact role of these two transcription factors in EN-NK/T-NT cases with aggressive features remains controversial. On the other hand, Lin *et al* (31) found that the expression of T-bet in EN-NK/T-NT cases was associated with reduced aggressive clinical features. Therefore, further investigation is required to determine the functions of these two transcription factors and their involvement in the presence and absence of EBV. Moreover, additional information regarding EBV stages (which was not performed in the present study), which can be obtained from peripheral blood EBV DNA, is required to further compare the difference between EBV stage 0 and negative EBV samples, with respect to the protein levels of T-bet. There was no significant difference between the expression levels of ETS-1 and T-bet and survival rate, which was consistent with our previous study (15).

In the present study, EBV was not detected using ISH in EN-NK-NT cases, which suggests that EBV may be a transient infection or there was no EBV infection present. The ISH method is the gold standard for EBV detection in clinical studies; however, another method, rarely used in daily diagnosis, proposed by Mundo *et al* (25) includes EBV microRNA detection, which is a sensitive method to identify the existence of EBV. It was found that EBV serves a causative role in the pathogenesis of Burkitt lymphoma and that the EBV genome may be lost following genetic changes, including DNA methylation and histone modifications involving E-cadherin and PYCARD gene loci, known as the 'hit and run' mechanism, which were not detectable using conventional ISH methods (20,34,35). This mechanism may also be used to understand the effects in EBV-negative NK/T-cell lymphoma. However, a case of intestinal aggressive NK-cell lymphoma, described by Martin *et al* (36), found that the EBV genome was not detectable. Tsuyama *et al* (23) also confirmed the lack of the EBV genome in EN-NK/T-NT using second-generation DNA sequencing analysis. Therefore, it was hypothesized that EBV infection was not present in some EN-NK/T-NT cases. However, due to sample quantity and quality, analysis of the EBV genome was not performed, and additional studies are required to increase the understanding of these atypical lymphomas and to further identify the characteristics of the disease spectrum.

It has been reported that there are numerous cytogenetic abnormalities in EN-NK/T-NT, including deletions of 1p, 6q, 11q, 13q and 17p, and gains of 1q, 2q, 7q, 17q and 20q (16,37), and a complex karyotype in a patient with EBV-negative EN-NK/T-NT has been reported by Gao *et al* (38), but no characteristic genetic abnormalities have been previously identified. The most common abnormality is the deletion of 6q21, and it has been hypothesized that this genetic alteration may serve a role in the occurrence and development of EN-NK/T-NT (16). However, whether this alteration plays an important role or is associated with disease progression has not been fully understood. Moreover, the downregulation of PRDM1 protein expression (via gene deletion, DNA methylation and/or microRNA aberrant expression), a tumor suppressor gene located on 6q21, has been considered as a potential candidate gene associated with the development of EN-NK/T-NT (16,39). The results from the present study suggests that there was negative PRDM1 expression (four cases) or weak expression (two cases) in the seven EBV-negative cases. Thus, it was hypothesized that PRDM1 may be a pathogenic gene, which is independent of EBV infection in EN-NK/T-NT, and is consistent with a previous study (16). The present study also identified differences in gene expression and PRDM1 protein expression, which may be due to the loss of heterozygotes of 6q21 and PRDM1 genes, in which there was still an undeleted allele in cells (16). Therefore, PRDM1 and 6q21 may play a pathogenic role, which is independent of EBV infection. However, further studies are required to analyze the changes in 6q21 and the PRDM1 gene, which may involve decreased or the loss of expression of PRDM1 via other pathogenic mechanisms, such as promoter methylation and microRNA inhibition (16,39). Our previous study found that the protein levels of PRDM1 were negatively correlated with T-bet or ETS-1 expression, and that it could interact with these two transcription factors to form a transcriptional regulatory network, which together regulates the growth and development of tumor cells (39); a similar association was also identified in the present study.

With the rapid development of high-throughput genomic and transcriptional analysis, progress has been made in identifying key cellular pathways underlying the dysregulation in EN-NK/T-NT, such as upregulation of JAK/STAT, RUNX3, PDGFRA, NOTCH1, Aurora kinase A and NF-κB-associated genes, and dysregulation of the c-Myc oncogene (11,40). Tsuyama *et al* (23) analyzed the gene expression profile of an EBV-negative EN-NK/T-NT case using the second generation sequencing method, and found mutations in KDM6A (V967G) and TP53 (G266R), which are commonly mutated in EBV-positive EN-NK/T-NT (41,42), suggesting that the epigenetic pathway of EBV-negative cases was similar to that of EBV-positive cases (36). Moreover, Gao *et al* (38) found that the PRC2 pathway may contribute to the development of EN-NK/T-NT. In EBV-negative cases, the activation of the PRC2 pathway may be associated with the upregulation of c-Myc, which then induces histone modification of H3K27me3 via the interaction with EZH2 and other molecules associated with PRC2, such as SUZ12 and EED, which is similar to other EBV-positive EN-NK/T-NT cases (38). In addition, other key genes, including genes encoding RNA helicase DDX3X, the JAK/STAT signaling pathway (JAK3, STAT3 and STAT5B) and tumor suppressors, such as TP53, MGA, PRDM1, protein tyrosine phosphatase κ, FOXO3, ATG, AIM and HACE1, have been identified (37). Furthermore, genes encoding RAS gene family and proto-oncogene (such as Myc), epigenetic modifiers (including KMT2D, MLL2, EP300, ASXL3 and ARID1A) and cell cycle and apoptotic regulators (including CDKN1A, CDKN2A, CDKN2B and FAS) have been identified (11,20,37,38). The pathogenicity of these genes may

be independent of EBV infection; however, this requires further investigation using large number of EBV-negative EN-NK/T-NT cases. The primary limitation of the present study was the inability to perform gene expression analysis on all tissues due to the small sample size.

In conclusion, the results from the present study may provide further evidence for the existence of EBV-negative EN-NK/T-NT cases; however, these cases remain rare. The clinicopathological characteristics of EBV-negative EN-NK/T-NT were found to be similar with those of EBV-positive cases. However, it was identified that more patients with EBV-negative EN-NK/T-NT were female, compared with patients with EBV-positive EN-NK/T-NT. In addition, two transcription factors, T-bet and ETS-1, were highly expressed in EBV-negative EN-NK/T-NT, while there was negative or weak expression of PRDM1. However, the present study did not demonstrate whether the prognosis of patients with EBV-negative expression was improved or worse compared with that in patients with EBV-positive expression. Therefore, understanding the EN-NK/T-NT EBV-negative variant is important for early diagnosis of this aggressive neoplasm. At present, previous studies have reported the mechanism of pathogenic genes, such as overexpression of EZH2 and trimethylated H3K27, overexpression and amplification of c-Myc, missense mutations of the STAT3 gene, strong expression of PD-L1 and CD30, in EBV-negative EN-NK/T-NT, and these genes could serve an important role in future targeted therapy (23,27,38,43). Moreover, future progress for the disease depends upon more robust diagnostic criteria with replicable molecular markers.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

TL designed the study. WW, LL, YZ and LN collected and analyzed the patient data. WW, LN and TL evaluated and interpreted the pathological and immunohistochemical results. YZ performed statistical analysis and interpreted the results. DL performed the immunohistochemical staining. XL was responsible for the technical operation of the ISH and FISH. WW, LN, LL and TL wrote the manuscript. LN, LL and TL revised the manuscript. All authors have read and approved the final version of the manuscript.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of Peking University First Hospital [approval no. 2013(571)].

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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